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14. ABSTRACT

Findings from this reporting period identify macrophages and efferocytosis as a key mediator of prostate cancer tumor growth. Specifically, phagocytic macrophages and efferocytosis were found to be upregulated in the blood of patients with metastatic prostate cancer. Moreover, inhibiting phagocytic macrophages with the chemotherapeutic trabectedin reduced efferocytosis and prostate cancer tumor size in murine models. In addition, trabectedin inhibition of macrophages significantly altered macrophages and efferocytosis in the bone microenvironment – a common site of prostate cancer metastasis. Furthermore, trabectedin treatment was found to significantly reduce bone mass and alter bone remodeling.

15. SUBJECT TERMS
Macrophages, Efferocytosis, Prostate Cancer, Bone

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Introduction

Regulatory T cells (Treg) are central to the maintenance of immunological self-tolerance and immune homeostasis. These cells are suppressors of aberrant or excessive immune responses. Th17 and induced Treg cells represent two T cell subsets that appear to have elements of shared lineage but ultimately bifurcate into distinct phenotypes with opposing activity, Th17 cells being proinflammatory and Tregs being anti-inflammatory. Dysregulated Th17 responses contribute to numerous autoimmune diseases (e.g. MS and IBD). Conversely, Treg activity restrains Th17-dependent autoimmunity and IBD. In the cancer setting, Treg are frequently increased in number and can restrain Th1-dependent anti-tumor responses. Recently Th17 responses were also shown to play a critical role in promoting carcinogenesis and the growth of established tumors.

The mechanisms regulating the balance between these functionally opposite lineages are just beginning to be elucidated. We recently found that hypoxia inducible factor 1 (HIF-1) plays a critical role in the balance between Treg/Th17 cells, and is indispensable in Th17 development (1, 2). We hypothesize that pathways of HIF-1 upregulation in T cells modulate cancer immunity and we predict that small molecule targeting of HIF-1 will undermine the processes supporting tumor initiation and progression. We proposed to test the efficacy of this novel treatment strategy in murine tumor models as either monotherapy or coupled with simultaneous Treg depletion to yield even more potent anti-tumor effects. Furthermore, we hope to elucidate the mechanisms of HIF-1 inhibition on T-cell response in a cancer setting. These therapeutic and mechanistic studies will hopefully allow us to validate the efficacy of HIF-1 inhibition in treatment of prostate cancer, and identify novel immune pathways and therapeutic targets for cancer therapy.

Key Words:

Hypoxia-inducible factor (HIF)-1; regulatory T cells (Treg); Th17 cells; Immunotherapy

Accomplishments:

What were the major goals of this project?

The major goals of this project include the three aims as follows:

I. Analyzing the consequences of genetic HIF-1 modulation or HIF1 inhibition on prostate-infiltrating lymphocytes and tumor progression in the Pro-HA x TRAMP Mouse Model;

II. Development of an in vivo HIF-1 reporter assay and correlation with Th17 cytokine production during tumor progression in the mice model;

III. Determine the efficacy of HIF-1 inhibitors in combinational modulation of the anti-tumor immune response, inhibition of tumor progression and augmentation of prostate cancer vaccines *in vivo*.

What was accomplished under these goals?

For this reporting period, we, in collaboration with Dr. Chuck Drake's laboratory at Hopkins have successfully developed a highly physiologically relevant mouse model for prostate cancer, namely pro-HA TRAMP model (3). Our pilot experiment showed that HIF1 inhibitor could slow down prostate tumor growth. As shown in Figure 1, ProHAxTRAMP mice ages 7 to 9 weeks were treated with vehicle (control group) or HIF1 inhibitors (Digoxin and Acriflavine). Ten weeks after the initial treatment, mice were sacrificed and tumor extent was evaluated as described previously (4). The net weight of the urogenital tract, a gross surrogate for tumor burden (5), was significantly decreased in HIF1 inhibitors treatment group (6, 7). While we have been waiting for greater expansion of the TRAMP mouse colony for testing the combinational therapy of HIF1 inhibition with Treg manipulation, we have further dissected the mechanism (s) by which HIF-1 inhibition affects T-cell response. These resounding, protective anti-tumor effects of HIF-1 blockade appear to result from multiple effects on T cells. Specifically we found that, in addition to elevating levels of immune-activating cytokines, interfering with HIF-1 also promotes the generation of a specialized tumor-killing class of T cells - the so-called "cytotoxic T lymphocytes" (CTLs) (manuscript in preparation). These results suggest that HIF-1 targeting during cancer will promote anti-tumor immunity by reshaping the T cell response on multiple levels. In order to better understand how HIF-1 targeting can reshape the CTL pool, we assessed the effects of HIF-1 deficiency on global CD8+ T cell gene expression using an RNAseq approach.

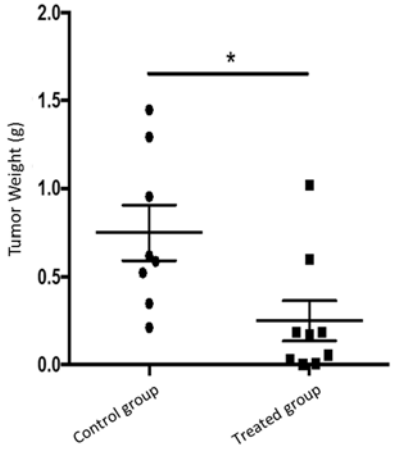

Figure 1. HIF1 inhibition could slow down prostate tumor growth. ProHAxTRAMP mice were treated with HIF1 inhibitors (Digoxin and Acriflavine, 2mg/kg for each drug) for 10 weeks before harvested for tumor weight analysis.

1. **RNAseq and network analysis reveals CD8 T cell gene expression in the setting of HIF-1 deficiency**

Metabolic factors and processes play a significant part in the shaping of T cell immune responses. In addition to being a key sensor and mediator of the cellular response to oxygen scarcity, HIF-1α is important for signaling induced by a number of metabolic and inflammatory stimuli. Previously we and others demonstrated a role for HIF-1α in driving Th17 differentiation. HIF-1 deficiency in T cells stunted the generation of IL-17-producing CD4+ T cells and instead favored the upregulation of Foxp3 (1, 2). Since metabolic factors and pathways have also been suggested to shape CD8+ T cell responses, we set out to determine the contribution of HIF-1 to these important agents of cell-mediated immunity.

To this end we utilized a whole transcriptome analysis approach (RNASeq) to observe changes in CD8+ T cell gene expression resulting from genetic HIF-1 modulation.

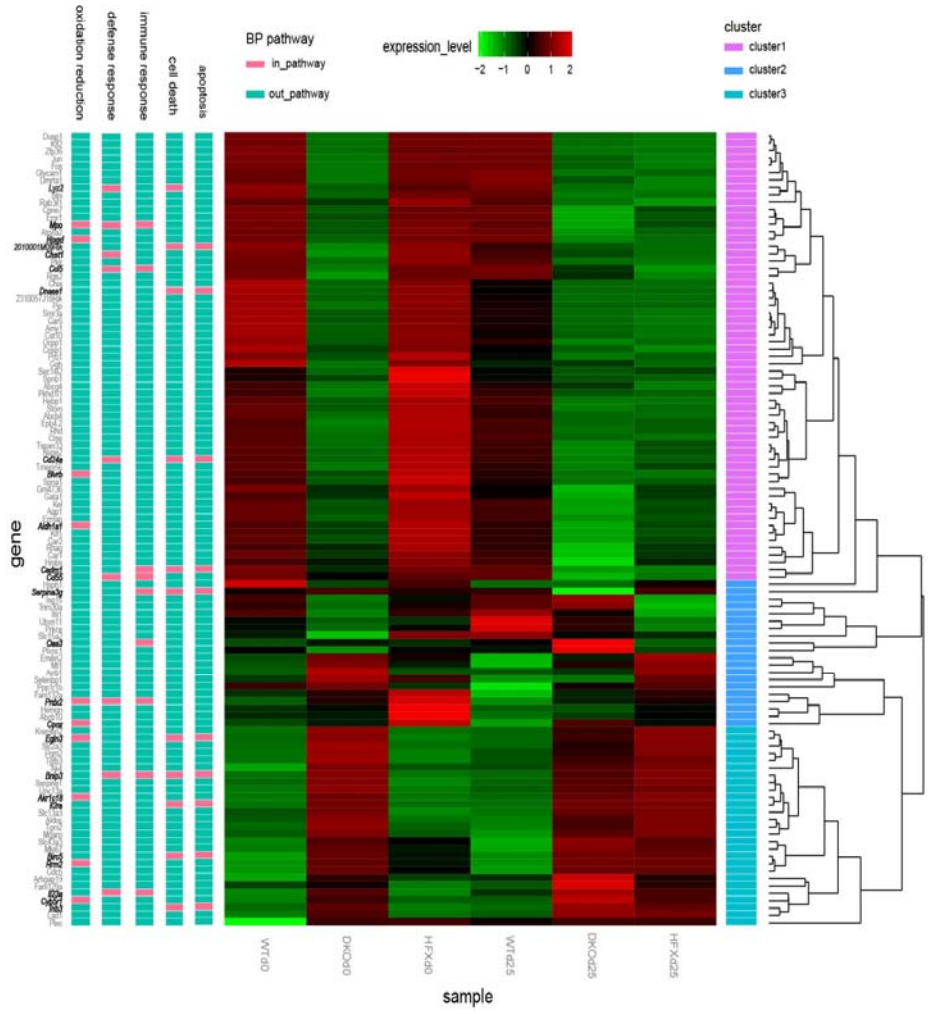

Figure 2. The heatmap of differentially expressed genes (with a cutoff of a q-value < 0.05) which are involved in the immune response and negative regulation of oxidation pathways by comparing among CD8+ T cells isolated from WT (wild type), DKO (HIF-1 and HIF-2 T-KO) and HFX(HIF-1 Tg) mice. Freshly isolated CD8+ T cells or those activated for 60 hrs in vitro were subject to RNAseq analysis.

As shown in Figure 2, RNA-seq analysis reveals that the differentially expressed genes are enriched in the pathways such as apoptosis, cell death and immune response. These results suggest that HIF-1 does play a role in shaping the CD8+ T cell response. Moreover, further analysis (Figure 3) suggests that HIF-1 may do so by modulating commitment to effector and memory CD8+ T cell subsets.

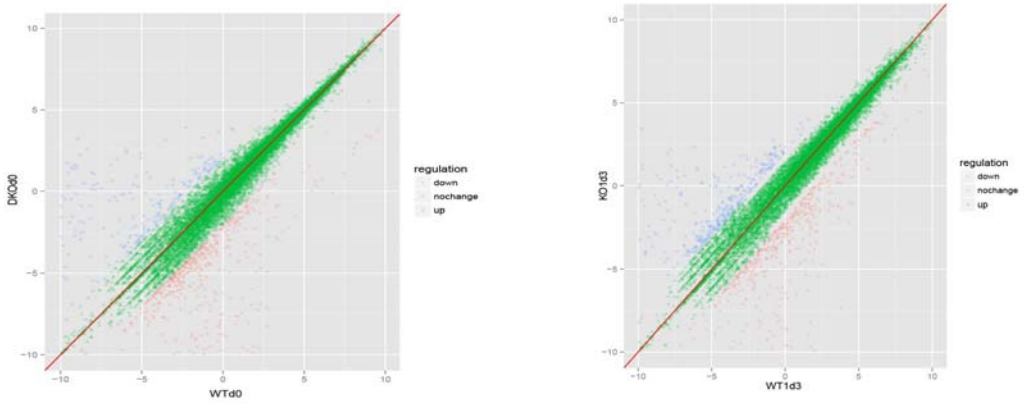

Figure 3. RNA-seq analysis of gene expression (Log2 expression level) by CD8+ T cells isolated from WT and DKO mice. The square (blue) and circle (red) in plots indicate the genes upregulated or downregulated in DKO cells relative to their expression in WT cells with a cutoff of a Log2 twofold change in expression (left). RNA-seq analysis of gene expression by CD8+ T cells isolated (from WT or KO mice) and cultured for 60 hrs in vitro in the presence of anti-CD3 and anti-CD28 (right).

2. HIF-1 deficiency induces a memory-like gene expression pattern in CD8+ T cells

As mentioned earlier, CD8+ cytotoxic T cells have important roles in the clearance of intracellular pathogens and tumors. Upon infection, CD8+ T cells transition from quiescent, poorly cytotoxic cells to metabolically active, proliferating cells with high cytolytic function and the capacity for rapid cytokine production. High glycolytic activity in CD8+ T cells has been linked to severely compromised generation of long-lived memory cells by driving T cells instead towards a terminally differentiated effector state. As shown in Figure 4, genetic down-modulation of HIF-1 indeed influences the differentiation of CD8+ effector and memory populations. RNAseq analysis reveals that more that 70% of memory signature genes (up and down-regulation) are expressed in HIF-1 deficient CD8+ T cells. Furthermore, we have proved that these HIF-1 deficient CD8+ T cells possess higher killing capacity compared to WT cells (data not shown).

Figure 4. HIF-1 deficiency induces signature genes expression used to predict the memory-precursor potential of CD8+ T cells

Summary: In summary, we have made considerable progress in our exploration of the utility of HIF-1 inhibition as a potential strategy for targeting prostate cancer in murine model. We have found that inhibition of HIF-1 with digoxin and acriflavine significantly inhibited tumor growth in the ProHAxTRAMP murine prostate cancer model. We also explored the potential mechanisms of HIF-1 mediated IFNγ suppression. Importantly, we have finished RNAseq analysis of WT vs. HIF-1 deficient CD8 T cells, and acquired a comprehensive grasp of HIF-1's impact on the genes expressed by potential tumor killing cells. Further bioinformatics analysis of the RNAseq dataset will lead to the identification of more potential immunotherapy targets. A manuscript reporting these and other exciting findings will be submitted in the coming months. In addition to our continued exploration of HIF-1's role in CD8 T cell differentiation, a collaborative effort carried out with the Quintana group at Harvard University has revealed a previously unknown role for HIF-1 in regulating the development of "Tr1" type Regulatory T cells. The results of this project were recently published in *Nature Medicine* (8).

B.3. What do you plan to do during the next reporting period to accomplish the goals?

We have made considerable progress in our evaluation of potential HIF-1 targeting therapies as promising strategies for bolstering anti-tumor immunity. While we have uncovered significant effects of HIF-1 deficiency beyond our original hypothesis, we will never the less continue to explore the HIF1 inhibition in combination with Treg targeting to combat the prostate cancer in mouse model (Aim3), and analyze the changes of prostate-infiltrating lymphocytes (Aim 1), which are important players at the interface of the tumor and the immune system. We will also make use of newly established HIF-1 reporter plasmid called HYPOXCR (Aim 2). Implementing this important asset will allow us to establish patterns of HIF-1 regulation both with the cryptic tumor microenvironment and elsewhere during cancer and at baseline. Hopefully we can use this HIF-1 reporter system to track HIF-1 activity in

diverse immune cells in the murine model as well as other proposed cancer models in coming years with continuing support from the DoD.

Since testing of HIF-1 inhibition as a potential strategy for combating prostate cancer gave very promising results, we will further explore the mechanisms behind these exciting findings. We have gained a lot knowledge for the RNAseq analysis. We plan to isolate the CD8 cells from the prostate-infiltrating lymphocytes from the prostate bearing mice received vehicle or HIF1 inhibitors by FLOW in next period of DoD support, followed by RNAseq analysis. This RNAseq study will help us identify pathways involved in tumor reduction as a result of HIF-1 inhibition, allowing us to identify additional layers of immune regulation, potentially culminating in the identification of novel therapeutic targets. Additionally we will explore additional combinational regiments including HIF-1 inhibition as ways to achieve even more effective anti-tumor effects (expanding on our original Aim 3).

IMPACT: Nothing to Report at this time

CHANGES/PROBLEMS: Nothing to Report at this time

PRODUCTS: Nothing to Report at this time

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

What individuals have worked on the project?

Name: Fan Pan
Role: PI
Contribution to Project: Dr. Pan has designed the proposed experiments, operates the lab in which they will be carried out, drafted the proposal and report, and analyzed data.
Other Funding Support: NIH

Name: Xuhao Ni, MD
Role: PhD candidate
Contribution to Project: Dr. Ni assisted with the design of experiments, carried out experiments, collected results and analyzed data.

Has there been a change in the other active support of the PD/PI(s) or senior/key personnel since the last reporting period? No

What other organizations have been involved as partners? No

SPECIAL REPORTING REQUIREMENTS: None

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